Phylogenomic analysis of *Pseudomonas nitroreducens* strains FY43 and FY47

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Abstract. Proper identification of strain is essential in understanding the ecology of a bacteria species. The classification of *Pseudomonas nitroreducens* is still being questioned and revised until now. The novel *P. nitroreducens* strains FY43 and FY47 used in this study have been reported to show a high level of tolerance to glyphosate. In this study, next-generation sequencing (NGS) and whole genome analysis were used to clarify the delineation of the species. Whole genome analysis showed that *P. nitroreducens* strains FY43 and FY47 shared high homology to five reference genomes of *P. nitroreducens*: strain B, Aramco J, NBRC 12694, DF05, and TX01. Phylogenomic and phylogenetic analysis (average nucleotide identity based on BLAST (ANIb), genome-to-genome distance (GGDC) analysis) showed that both *P. nitroreducens* strains FY43 and FY47 are *Pseudomonas nitroreducens* members. However, strains DF05 and TX01 were not correctly assigned at the species level for all the analyses. The *P. nitroreducens* strain DF05 and TX01 should be further investigated for their classification as the correct species classification is the prerequisite for future diversity studies.

Keywords: ANIb, GGDC, MLSA, Next-generation Sequencing, *Pseudomonas nitroreducens*

INTRODUCTION

Pseudomonas is a genus of rod-shaped and gram-negative bacteria (Euzéby, 1997). There are 216 species of *Pseudomonas* sp., including 18 subspecies, which have been recorded in the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2014). *Pseudomonas* sp. can survive actively in water, soil and plants.

Identification and classification of *Pseudomonas* strains is a necessary step to understand the ecology of this genus. In the early years, *Pseudomonas* sp. was classified based on their phenotypes, such as nutritional screening (Stanier *et al.*, 1966; Novik *et al.*, 2015). In 1984, the taxonomy of the *Pseudomonas* genus was revised when using molecular techniques, such as DNA-DNA hybridization (DDH) and rRNA-DNA hybridization (Gomila *et al.*, 2015). Gomila *et al.*

(2015) found that only 31 out of 96 previously approved *Pseudomonas* sp. were considered as true species in the list of *Pseudomonas* sp. However, DDH values may not be reproducible as it is a laboratory-based technique, which may vary depending on the experimental settings and laboratory methods employed (Colston *et al.*, 2014). Moreover, DDH requires a large amount of high-quality DNA, which could be labor intensive and time-consuming (Goris *et al.*, 2007). The data generated from the DDH technique cannot be archived and shared between laboratories (Colston *et al.*, 2014). Therefore, the DDH data might not be suitable for new species description (Goris *et al.*, 2007; Colston *et al.*, 2014).

Currently, 16S rRNA gene sequencing is commonly used for bacterial classification.

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However, this method is often questioned due to insufficient resolution for specific species identification (Mulet *et al.*, 2010). To enhance the phylogenetic resolution, a few reference genes are often used as phylogenetic molecular markers. This is because these reference genes are highly conserved and have a high degree of sequence divergence, especially at the genus level (Glaeser & Kämpfer, 2015). However, a single gene-based sequence does not reflect the accurate phylogenetic relationship. Hence, a multiple gene-based phylogenetic analysis has been developed to eliminate the bias created from the single gene-based sequence analysis. Multilocus sequence analysis (MLSA) was introduced by Maiden *et al.* (1998). This method involves concatenating several reference gene fragments to build a phylogenetic tree based on fragment similarities. Four reference genes, namely 16S rRNA, *rpoB*, *rpoD*, and *gyrB*, have been proven reliable in strain classification and species delineation for *Pseudomonas* sp. (Mulet *et al.*, 2013; Garrido-Sanz *et al.*, 2017).

Next-generation sequencing (NGS) is a cost-effective sequencing method for comparative genomic study. It can produce high-quality whole genome sequences, which is useful for taxonomic relationships and evolutionary studies (Chain *et al.*, 2009; Gomila *et al.*, 2015). With NGS, new taxa could be discovered, and readily built genomic data could be shared among researchers (Colston *et al.*, 2014). Two bioinformatics tools, namely genome-to-genome distance calculator (GGDC) and average nucleotide identity (ANI), have been developed for NGS analysis. GGDC is a substitution for DDH, whereby it gives values that are comparable to experimental-based DDH values (Meier-Kolthoff *et al.*, 2013). ANI calculates the mean of similarity values between two homologous genomic sequences. It is now a new standard for microbial species classification (Konstantinidis *et al.*, 2006). Previous studies demonstrated that species identification was improved by combining MLSA or analysis based on common genes in the genus with the ANI tool (Chan *et al.*, 2012; Tarazona *et al.*, 2014). To date, many *Pseudomonas* strains have been studied and classified, but the correct taxonomic position remains uncertain. For instance, *Pseudomonas oryzzihabitans* was omitted from the classification of *P. psychrophila* when analyzed using the DDH

method (Yumoto *et al.*, 2001). However, the recent re-classification of both species was conducted using phylogenetic analysis, MLSA (four housekeeping genes, 16S rRNA, *gyrB*, *rpoB* and *rpoD* were included), and phylogenomic analysis (ANiB and GGDC) (Lalucat *et al.*, 2020). The analysis showed that both species are the same genomospecies (Lalucat *et al.*, 2020).

Mulet *et al.* (2013) re-classified *Pseudomonas putida* strains based on gene sequences of 16S rRNA, *gyrB*, and *rpoD*. The authors found that some strains previously classified as *Pseudomonas putida* were phylogenetically affiliated with other closest relatives in the same genus (Mulet *et al.*, 2013). In total 29 genomes designated as *Pseudomonas fluorescens* may be misclassified as they are also found in 20 different genomic clusters (Jun *et al.*, 2016). Therefore, a thorough taxonomic analysis is needed to assist comparative genomic analysis to avoid confusion in the identification and classification of *Pseudomonas nitroreducens*.

In this study, we generated high-quality draft genome sequences of *Pseudomonas nitroreducens* strains FY43 and FY47 using various genome assemblers. Furthermore, together with *P. nitroreducens* in this study and previously reported strains, we re-classified the *P. nitroreducens* species through phylogenomic approaches.

MATERIALS AND METHODS

Whole genome sequencing and assembly for *Pseudomonas nitroreducens* strains FY43 and FY47

Pseudomonas nitroreducens strains previously reported by Looi (2016) and Tan *et al.* (2020) were used in this study. The genomic DNA of FY43 and FY47 isolates were extracted using EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, China) according to the manufacturer's protocol. The extracted genomic DNA was sent for whole genome sequencing using NGS service provided by Apical Scientific Sdn. Bhd. and Next Gene Scientific Sdn. Bhd. The NGS libraries were prepared using VAHTS Universal DNA library prep kit for Illumina (Vazyme Biotech, China). Genome sequencing was performed using a high-throughput Illumina

HiSeq X Ten technology platform with a 150 bp paired-end strategy. Sequencing reads were pre-processed by removing poor quality reads (e.g. Phred score ≤ 20) and ambiguous base calls (Ns) using Cutadapt (version 1.9.1). The post-processed clean reads were subjected to *de novo* genome assembly using different assembly software, such as SPAdes (Bankevich *et al.*, 2012), ABySS (Simpson *et al.*, 2009), and Velvet assembler (Zerbino & Birney, 2008). The best assembly/scaffold was chosen based on N50 values, number of contig/scaffold, and assembly/scaffold size. The assembled genome of FY43 and FY47 was submitted to NCBI GenBank under BioProject ID PRJNA779109.

Phylogenetic inferences

A total of 8 *Pseudomonas* strains, namely 5 *Pseudomonas nitroreducens* strains (NBRC12694, Aramco J, B, FY43 and FY47), 2 *Pseudomonas* sp. strains DF05 and TX1 (originally classified as *P. nitroreducens*), and *Pseudomonas denitrificans* ATCC13867 (as an outgroup), were used to construct a phylogenetic tree based on four reference genes from each strain, namely 16S rRNA, *gyrB*, *rpoB*, and *rpoD*. The nucleotide sequences of each reference gene were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and constructed using the Maximum Likelihood method with 500 bootstrap replicates. The gene sequences of the 16S rRNA, *gyrB*, *rpoB*, and *rpoD* were then concatenated for the multilocus sequence analysis. The construction of the supermatrix-based tree was the same as the single gene-based tree (Gomila *et al.*, 2015).

Average nucleotide identity (ANI) and genome-to-genome distance (GGDC)

The average nucleotide identity based on the BLAST algorithm (ANiB) was calculated between genome pairs for each strain using JSpecies (Richter & Rosselló-Móra, 2009). The genomes (the query) were cut into 1,020 nucleotide fragments before calculating the ANiB value between the query genome and the reference genome. The ANiB represents a mean identity of all the BLASTN matches showing more than 30% overall sequence identity over an alignable region of at least 70% coverage of their length. GGDC was calculated using the web service <http://ggdc.dsmz.de> (Meier-Kolthoff *et al.*, 2013).

RESULTS AND DISCUSSION

De novo genome assembly of *Pseudomonas nitroreducens* strains FY43 and FY47

The genomes of *Pseudomonas nitroreducens* strains FY43 and FY47 were sequenced and assembled *de novo*. The draft genome sizes of strains FY43 and FY47 were 6,255,176 and 6,266,999 bp, respectively (Supplementary Data). The contig number of FY43 and FY47 were 68 and 74, respectively. The assembled genome sizes of both *Pseudomonas nitroreducens* strains FY43 and FY47 in this study were in accordance with the genome sizes of *Pseudomonas* reported by Thakur *et al.* (2016) and Molina-Mora *et al.* (2020). According to the *de novo* assembly results from the previous studies, the genome sizes of *Pseudomonas* sp. were in the range of 5,098,132 to 7,190,208 bp, whereas the contig numbers were in between 86 to 684 (Thakur *et al.*, 2016; Molina-Mora *et al.*, 2020). In this study, the results showed that ABySS assembly software (Table 1) generated the largest genome size, highest N50 values, and lowest contig number compared to SPAdes and Velvet assemblers. Hence, ABySS assembly software was selected for the subsequent analysis.

Table 1. *De novo* genome assembly of *Pseudomonas nitroreducens* strains FY43 and FY47 analyzed from ABySS assembly software.

ABySS	FY43	FY47
Genome Size	6,255,176	6,266,999
Contig No	68	74
N50	239,855	240,409
Minimum contig	204	204
Maximum contig	858,264	863,737

Reclassification of *Pseudomonas nitroreducens* species based on phylogenetic analysis

Phylogenetic analysis was conducted to clarify the delineation of *Pseudomonas nitroreducens* species. In this study, the sequences of 16S rRNA, *gyrB*, *rpoB*, and *rpoD* from each strain were evaluated using a phylogenetic tree. Based on the 16S rRNA, *Pseudomonas nitroreducens* strains FY43 and FY47 were grouped with other *Pseudomonas nitroreducens* species, except DF05, TX01, and *Pseudomonas denitrificans* ATCC13867 (Figure 1). The finding suggests that DF05 and TX01 might not belong to *Pseudomonas nitroreducens* species. To further confirm this finding, phylogenomic analysis and MLSA on other genes, namely *gyrB*, *rpoB*, and *rpoD*, were performed. Interestingly, all trees

revealed that *P. nitroreducens* strains FY43 and FY47 were grouped with *P. nitroreducens* strain B, Aramco J, and NBRC 12694, whereas *Pseudomonas* sp. strains DF05 and TX01 were clearly separated from other strains (Figures 1 to 4). Phylogenetic analysis using *rpoD* gene showed that *Pseudomonas* sp. DF05 and TX01 formed a subclade that separated them from the rest of *Pseudomonas nitroreducens* species (Figure 4). Although the *rpoD* gene only grouped *Pseudomonas* strains into 2 main clades, it is still suitable and widely adopted for genotyping *Pseudomonas* genera (Mulet *et al.*, 2009). These findings showed that *Pseudomonas* sp. strains DF05 and TX01 might be misclassified as *Pseudomonas nitroreducens*.

Figure 1. Phylogenetic tree of 8 *Pseudomonas* strains based on 16S rRNA gene sequences.

Figure 2. Phylogenetic tree of 8 *Pseudomonas* strains based on *gyrB* gene sequences.

Figure 3. Phylogenetic tree of 8 *Pseudomonas* strains based on *rpoB* gene sequences.

Figure 4. Phylogenetic tree of 8 *Pseudomonas* strains based on *rpoD* gene sequences.

Figure 5. Phylogenetic tree of 8 *Pseudomonas* strains inferred by MLSA.

Reclassification of *Pseudomonas nitroreducens* species based on phylogenomic analysis

To further confirm the identity of *Pseudomonas nitroreducens* strains FY43 and FY47, as well as *Pseudomonas* sp. strains DF05 and TX01, genomic analysis, including (i) ANIb, (ii) analysis based on MLSA, and (iii) GGDC, were utilized.

Average nucleotide identity based on BLAST (ANIb) analysis

The strains compared are classified as different species if the ANIb value is below 95%, whereas above 95% indicates that there are of the same species (Garrido-Sanz *et al.*, 2016). *Pseudomonas nitroreducens* strains FY43 and FY47 have a similarity lower than 95% with *Pseudomonas* sp. strains DF05 and TX01. The ANIb values for *P. nitroreducens* strains FY43 and FY47 were above 95% when comparing with reference *P. nitroreducens* strains B, Aramco J, and NBRC 12694 (Figure 6). This depicted that *Pseudomonas* sp. strains DF05 and TX01 belong to the same species but different from *P. nitroreducens* strains B, Aramco J, NBRC 12694, FY43, and FY47.

ANIb matrix was generated based on *Pseudomonas nitroreducens* strain B, Aramco J, NBRC 12694, FY43, and FY47, *Pseudomonas* sp. strains DF05 and TX01, as well as *Pseudomonas denitrificans* strain ATCC13867 (highest similarity with *Pseudomonas* sp. strains DF05 and TX01 in accordance to results from BLASTN). *P. nitroreducens* strains B, Aramco J, NBRC 12694, FY43, and FY47 exhibit a pairwise ANIb of more than 95%, clustering them into the same species (Figure 7). *Pseudomonas* sp. strains DF05 and TX01 showed low ANIb value (<95%) to all *Pseudomonas* sp., including *P. nitroreducens* strains B, Aramco J,

NBRC 12694, FY43, FY47, and *Pseudomonas denitrificans* strain ATCC13867, indicating that *Pseudomonas* sp. strains DF05 and TX01 do not belong to *Pseudomonas nitroreducens* or *Pseudomonas denitrificans*.

Genomic analysis based on MLSA

The calculations based on MLSA analysis were carried out to provide a better resolution for species delineation. Strain clustering in MLSA was maintained at a cut-off of 97% (Gomila *et al.*, 2017). In this study, the MLSA value of *P. nitroreducens* strains FY43 and FY47 was more than 97%, whereas *Pseudomonas* sp. strains DF05 and TX01 were lower than 97% (Figure 8). Moreover, the MLSA value of *P. nitroreducens* strains FY43 and FY47 in comparison with reference *P. nitroreducens* strains B, Aramco J, and NBRC 12694 was more than 97% (Figure 8). This confirmed that *Pseudomonas* sp. strains DF05 and TX01 do not belong to *Pseudomonas nitroreducens*.

Genome-to-genome distance (GGDC) analysis

The GGDC similarities were calculated between pairwise genomic comparisons to clarify phylogenetic assignments to species. The GGDC values utilize a specific distance formula, where the similarity of species is cut-off at 70% (Gomila *et al.*, 2015). When comparing *Pseudomonas nitroreducens* strains FY43 and FY47 to reference *P. nitroreducens* strains B, Aramco J, and NBRC 12694, we found that their GGDC values were more than 70% (Figure 9). Similar to ANIb and MLSA analysis, *Pseudomonas* sp. strains DF05 and TX01 recorded less than 70% GGDC. This result further confirmed that *Pseudomonas* sp. strains DF05 and TX01 may not be correctly assigned.

Figure 6. Genomic analysis of *Pseudomonas* sp. using ANIb analysis.

Figure 7. The ANIb matrix of 8 *Pseudomonas* genomes from *P. nitroreducens* strains FY43, FY47, B, NBRC12694, Aramco J, *Pseudomonas* sp. strains DF05, TX01, and *Pseudomonas denitrificans* strain ATCC13867.

Figure 8. Genomic analysis of *Pseudomonas* sp. using MLSA analysis.

Figure 9. Genomic analysis of *Pseudomonas* sp. using GGDC analysis.

CONCLUSION

In this study, the genome of *Pseudomonas nitroreducens* strains FY43 and FY47 were extracted and sequenced using NGS approach. Our results indicated that ABySS assembly software can generate larger genome size, higher N50 values, and lower contig numbers compared to SPAdes and Velvet assemblers. The phylogenetic trees (16S rRNA, *gyrB*, *rpoB*, *rpoD* and MLSA) and phylogenomic analysis (ANIb, MLSA and GGDC method) confirmed that *P. nitroreducens* strains FY43 and FY47 are *Pseudomonas nitroreducens*.

Pseudomonas sp. strains DF05 and TX01, however, was not classified as the same species as *P. nitroreducens* strains B, Aramco J, NBRC 12694, FY43, and FY47. Therefore, the species delineation of *Pseudomonas* sp. strains DF05 and TX01 should be revised.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interests exists.

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